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(54) Nucleotide Sequences for Bovine Sex Determination

(72) Wildeman, Alan G. - Canada ;  
Kelly, John J. - Canada ;

(71) University of Guelph - Canada ;

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ABSTRACT OF THE DISCLOSURE

The invention relates to purified and isolated nucleotide sequences from the Y chromosome and autosomal chromosomes of bovines. Single strand DNA hybridization primers are provided which hybridize to the Y chromosome or autosomal sequences and which can be used to detect the presence of Y chromosome and autosomal DNA respectively. The nucleotide sequences may be used to determine the sex of bovines. Thus the invention also relates to methods of detecting the presence of Y chromosomal DNA and autosomal DNA respectively; and to methods of determining the sex of a bovine cell using the nucleotide sequences and hybridization primers of the invention.

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BP File No. 6580-003/SMcD

**Title: NUCLEOTIDE SEQUENCES FOR BOVINE SEX DETERMINATION**

**FIELD OF THE INVENTION**

The invention relates to novel nucleotide sequences  
5 isolated from the Y chromosome and autosomal DNA of  
bovines, and fragments thereof; hybridization primers  
which are capable of hybridizing with the novel sequences;  
use of the sequences and hybridization primers to detect  
the presence of Y chromosomal DNA and autosomal DNA  
10 respectively; methods of detecting the presence of Y  
chromosomal and autosomal DNA using the sequences and  
hybridization primers of the invention; and methods of  
determining the sex of a bovine cell using the sequences  
and hybridization primers of the invention.

**15 BACKGROUND OF THE INVENTION**

Y specific probes constructed from DNA sequences specific  
to the bovine Y chromosome have been used to detect the  
sex of animal cells, for example from bovine  
preimplantation embryos (see, for example Ellis, U.S.  
20 Patent No. 4,769,319; Kwoh and Gingeras, PCT Application  
No. US90/03318; Herr et al, A. & N.Z. Soc. Study Cell  
Biol. Proc. 1989; Higashi et al, Jpn. J. Anim. Reprod.  
37:115, 1991; and Miller and Koopman, Animal Genet. 21:77,  
1990).

25 Several primers have been identified which have some  
degree of specificity for bovine Y chromosomes, and which  
can be used in a polymerase chain reaction (PCR) to  
provide an indication of the sex of bovine embryo cells  
(Kwoh and Gingeras, PCT Application No. US90/03318; Reed  
30 et al, PCT Application No. AU89/0029; Herr et al, A. & N.Z.  
Soc. Study Cell Biol. Proc. 1989; Bredbacka et al, Reprod.  
Dom. Anim. 26:75, 1991; Miller, Reprod. Dom. Anim. 26:58,  
1991; Glasgow, New Scientist 9:31, 1989; Schwerin et al,  
Reprod. Dom. Anim. 26:70, 1991; and Higashi et al, Jpn. J.  
35 Anim. Reprod. 37:115, 1991). Bovine sexing by PCR depends  
on the selective amplification of a DNA sequence only

found in male embryos i.e. on the Y chromosome. Thus male cells are recognised on the basis of amplification of the male specific DNA sequence, and female cells are recognised by the absence of an amplification reaction.

5 Accurate sex determination by PCR amplification depends  
on the specificity of the Y-specific hybridization primers  
and on a reliable control reaction which allows a true  
negative result (female) to be distinguished from cases  
where the PCR reaction is inadequate for some extraneous  
10 reason, such as lack of DNA in the sample, faulty reagents  
or inappropriate reaction conditions. The simultaneous  
amplification of an autosomal sequence has been used as a  
control for amplification (Kwoh and Gingeras, PCT  
Application No. US90/03318; Reed et al, PCT Application  
15 No. AU89/0029; Herr et al, A. & N.Z. Soc. Study Cell Biol.  
Proc. 1989; Bredbacka et al, Reprod. Dom. Anim. 26:75,  
1991; and Schwerin et al, Reprod. Dom. Anim. 26:70,  
1991).

In order to be commercially viable and useful, it is  
20 desirable that a method of sex determination be as close  
to 100% accurate as possible. The accuracy of PCR sexing  
of embryonic cells depends upon the interplay of a number  
of factors, including the sensitivity and specificity of  
the assay. These factors depend to a large extent on the  
25 stringency of the Y-specific hybridization primers for Y-  
specific DNA and the complete lack of a reaction with any  
non Y-specific DNA. It is also advantageous for the  
autosomal control reaction to be reliable and easily  
distinguishable from a positive male reaction.

30 **SUMMARY OF THE INVENTION**

The present inventors have identified novel nucleotide  
sequences highly specific for the Y chromosome and  
autosomal DNA of bovines which permit the accurate  
determination of the sex of bovine cells, particularly  
35 from embryos or fetuses.

The present invention provides a purified and isolated nucleotide sequence comprising the sequence Y1 shown in SEQ ID NO:3 and Figure 3; Y2 shown in SEQ ID NO:6 and Figure 4; or Y3 shown in SEQ ID NO:9 and Figure 5.

5 The invention relates to a purified and isolated nucleotide sequence comprising the sequence Y1 shown in SEQ ID NO:3 and Figure 3; Y2 shown in SEQ ID NO:6 and Figure 4; or Y3 shown in SEQ ID NO:9 and Figure 5, which is capable of hybridizing to a significantly greater  
10 extent with Y chromosomal DNA than with autosomal or X chromosomal DNA.

The invention also relates to a purified and isolated nucleotide sequence having substantial homology to the sequence Y1 shown in SEQ ID NO:3 and Figure 3; Y2 shown in SEQ ID NO:6 and Figure 4; or Y3 shown in SEQ ID NO:9 and Figure 5, which is capable of hybridizing to a significantly greater extent with Y chromosomal DNA than with autosomal or X chromosomal DNA.

The invention further provides fragments of the above sequences, including any contiguous portion of the above sequences consisting of 15 or more nucleotides, preferably comprising the sequence 5-1 or 6-1 shown in SEQ ID NO:4 and SEQ ID NO:5 respectively and Figure 3; K3 or K4 shown in SEQ ID NO:7 and SEQ ID NO:8 respectively and Figure 4; 25 or K1 or K2 shown in SEQ ID NO:10 and SEQ ID NO:11 respectively and Figure 5.

The invention further provides a purified and isolated nucleotide sequence having the sequence AUTO 1 shown in SEQ ID NO:12 and Figure 6, or AUTO 2 shown in SEQ ID NO:15 and Figure 7, or a sequence having substantial homology thereto which is capable of hybridizing to a significantly greater extent with autosomal DNA than with Y chromosomal DNA. The invention also relates to fragments, or any contiguous portion consisting of 15 or more nucleotides, 35 of the sequence AUTO 1 shown in SEQ ID NO:12 and Figure 6, or AUTO 2 shown in SEQ ID NO:15 and Figure 7, or a

sequence having substantial homology thereto which is capable of hybridizing to a significantly greater extent with autosomal DNA than with Y chromosomal DNA.

The invention further provides fragments of the above-mentioned nucleotide sequences, preferably comprising the sequences JC4 and JC7 shown in SEQ ID NO:13 and SEQ ID NO:14 respectively and Figure 6 or JK1 and JK2 shown in SEQ ID NO:16 and SEQ ID NO:17 respectively and Figure 7, or sequences having substantial homology thereto.

- 10 The invention also relates to a method of detecting the presence of autosomal DNA or Y chromosomal in a tissue or cell sample, preferably a bovine tissue or cell sample, most preferably from a member of the genus *Bos*, using fragments of the above-mentioned nucleotide sequences.
- 15 The invention also relates to a method of determining the sex of a bovine cell by isolating a DNA sample from the cell; treating the sample with one or more pairs of Y-specific hybridization primers, which are capable of amplifying a nucleotide sequence Y1 shown in SEQ ID NO:3 and Figure 3; Y2 shown in SEQ ID NO:6 and Figure 4; or Y3 shown in SEQ ID NO:9 and Figure 5, or a sequence having substantial homology thereto, under hybridization conditions in the polymerase chain reaction to produce amplified product and; detecting the amplified product,
- 20 25 whereby the sex of the cell is determined as male by the presence of amplified product and female by the absence of amplified product.

In a preferred embodiment, the invention relates to a method of determining the sex of a bovine cell, preferably a bovine cell from an embryo or amniotic fluid using fragments of the Y specific nucleotide sequences of the invention as hybridization primers, which method comprises obtaining a DNA sample from the cell; treating the sample with one or more pairs of hybridization primers selected from the group consisting of the sequences 5-1 and 6-1

shown in SEQ ID NO:4 and SEQ ID NO:5 respectively and Figure 3; K3 and K4 shown in SEQ ID NO:7 and SEQ ID NO:8 respectively and Figure 4; or K1 and K2 shown in SEQ ID NO:10 and SEQ ID NO:11 respectively and Figure 5, under 5 hybridization conditions in the polymerase chain reaction to produce amplified product, and; detecting the amplified product, whereby the sex of the cell is determined as male by the presence of amplified product and female by the absence of amplified product.

- 10 In a particularly preferred embodiment, the DNA sample is simultaneously treated, as a control, with one or more pairs of hybridization primers comprising the fragments JC4 and JC7 shown in SEQ ID NO:13 and SEQ ID NO:14 respectively and Figure 6 or JK1 and JK2 shown in SEQ ID
- 15 NO:16 and SEQ ID NO:17 respectively and Figure 7, or sequences having substantial homology thereto, which are capable of hybridizing to a significantly greater extent with autosomal DNA than with Y chromosomal DNA, whereby the presence of bovine DNA and hybridization conditions
- 20 are confirmed by the presence of autosomal amplification product.

In a further preferred embodiment the invention provides a kit for determining the sex of a bovine cell comprising a pair of hybridization primers which are complementary to 25 a fragment of the nucleotide sequence Y1 shown in SEQ ID NO:3 and Figure 3, Y2 shown in SEQ ID NO:6 and Figure 4; or Y3 shown in SEQ ID NO:9 and Figure 5, and which are capable of hybridizing to a significantly greater extent with Y chromosomal DNA than with autosomal or X 30 chromosomal DNA under hybridization conditions in the polymerase chain reaction (PCR) to produce a Y chromosomal amplification product; the reagents required for the PCR reaction and; means for detecting the amplification product.

- 35 In a particularly preferred embodiment, the invention provides a kit for carrying out the method of sexing

bovine cells, comprising a pair of hybridization primers, preferably 5-1 and 6-1 shown in SEQ ID NO:4 and SEQ ID NO:5 respectively and Figure 3; K3 and K4 shown in SEQ ID NO:7 and SEQ ID NO:8 respectively and Figure 4; or K1 and 5 K2 shown SEQ ID NO:10 and SEQ ID NO:11 respectively and in Figure 5 or sequences having substantial homology thereto, which under hybridization conditions in the polymerase chain reaction (PCR) amplify a Y chromosomal product, means for detecting the product and, the reagents 10 required for the PCR reaction. Preferably, the kit is further composed of a pair of hybridization primers, most preferably the further pair of hybridization primers have the sequences JC4 and JC7 shown in SEQ ID NO:13 and SEQ ID NO:14 respectively and Figure 6 or JK1 and JK2 shown in 15 SEQ ID NO:16 and SEQ ID NO:17 respectively and Figure 7, or sequences having substantial homology thereto, which amplify an autosomal sequence.

The invention also relates to a method of detecting the presence of Y chromosomal DNA in a tissue or cell sample, 20 preferably a tissue or cell sample from a bovine, most preferably from a member of the genus Bos, comprising selecting one or more fragments of the invention which are capable of hybridizing to a significantly greater extent with Y chromosomal DNA than with autosomal or X 25 chromosomal DNA, contacting the sample under hybridization conditions with one or more of the fragments which are labelled with a detectable marker and determining the degree of hybridization between the DNA of the sample and the fragment.

30 **DESCRIPTION OF THE DRAWINGS**

The invention will now be described in relation to the drawings in which:

Figure 1 shows the hybridization primers used to amplify the 157 bp segment of bovine male DNA, BOV97M;

35 Figure 2 shows the restriction map of the Eco RI fragment of the 20 kb segment of male bovine DNA;

Figure 3 shows the DNA sequence of Y1;

Figure 4 shows the DNA sequence of Y2;  
Figure 5 shows the DNA sequence of Y3;  
Figure 6 shows the DNA sequence of AUTO1;  
Figure 7 shows the DNA sequence of AUTO2; and  
5 Figure 8 is a diagrammatic illustration of a gel  
showing the results of PCR amplification for the  
determination of the sex of bovine cells.

DETAILED DESCRIPTION OF THE INVENTION

As hereinbefore mentioned, the present invention relates  
10 to a purified and isolated nucleotide sequence comprising  
the sequence Y1 shown in SEQ ID NO:3 and Figure 3; Y2  
shown in SEQ ID NO:6 and Figure 4; or Y3 shown in SEQ ID  
NO:9 and Figure 5, or a sequence having substantial  
homology thereto, which is capable of hybridizing to a  
15 significantly greater extent with Y chromosomal DNA than  
with autosomal or X chromosomal DNA. The invention  
provides fragments of the above sequences, preferably  
comprising the sequence 5-1 or 6-1 shown in SEQ ID NO:4  
and SEQ ID NO:5 respectively and Figure 3; K3 or K4 shown  
20 in SEQ ID NO:7 and SEQ ID NO:8 respectively and Figure 4;  
or K1 or K2 shown in SEQ ID NO:10 and SEQ ID NO:11  
respectively and Figure 5.

It will be appreciated that the invention includes  
nucleotide sequences which have substantial sequence  
25 homology with the above-mentioned nucleotide sequences  
shown in the Figures. The term "sequences having  
substantial sequence homology" used herein means those  
nucleotide sequences which have slight or inconsequential  
sequence variations from the sequences disclosed in SEQ ID  
30 NOS: 1 to 17 and in Figures 1, and 3-7, i.e. the  
homologous sequences function in substantially the same  
manner. It is understood that nucleic acid molecules can  
tolerate a certain number of mismatched base pairs and  
still form duplexes. Accordingly a degree of mismatch can  
35 be tolerated in a hybridization reaction. The variations  
may be attributable to local mutations or structural  
modifications.

It will also be appreciated that a double stranded nucleotide sequence comprising a DNA segment of the invention or an oligonucleotide fragment thereof, hydrogen bonded to a complementary nucleotide base sequence, an RNA made by transcription of this doubled stranded nucleotide sequence, and an antisense strand of a DNA segment of the invention, an oligonucleotide fragment encoded by the DNA segment, or a complementary sequence of a DNA sequence of the invention or fragment thereof, are contemplated within 10 the scope of the invention.

The nucleotide sequences of the invention can be prepared, for example, by the method generally described below and particularly described in the examples.

Sequences from the Y chromosome of bovines may be 15 identified by screening a bovine male genomic library with a known sequence from the Y chromosome, for example BOV97M described by Miller and Koopman (Animal Genetics, 21:77, 1990). A bovine male genomic library may be constructed in the vector lambda FIX II (Stratagene) using a 20 preparation of total male bovine DNA. The term "male bovine DNA" refers to DNA extracted from male bovine tissue, preferably the liver.

The bovine male genomic library may be screened with the Y chromosome specific DNA probe BOV97M. This probe may be 25 obtained from male bovine DNA, for example by PCR amplification. Preferably, the BOV97M sequence may be amplified by the pair of primers shown in SEQ ID NOS:1 and 2 and in Figure 1, which are complementary to the ends of the 157 bp segment BOV97M.

30 After PCR amplification, the BOV97M fragment may be ligated into a cloning vector, preferably pT218U (Pharmacia), and transformed into a eukaryotic or prokaryotic cell, preferably a bacterium, most preferably *E. coli*. The BOV97M fragment may be prepared in quantity 35 and radiolabelled by nick translation. It will be

appreciated that the term "nick translation" refers to the incorporation of deoxynucleoside triphosphates (dNTPs), preferably radiolabelled, at nicks introduced into a nucleotide sequence by limited treatment with DNase I. It  
5 will also be appreciated that other techniques for labelling nucleotides are known in the art.

The radiolabelled BOV97M probes may be used to screen a bovine male genomic library and to identify sequences of Y chromosomal DNA, preferably a 20 kb sequence of Y  
10 chromosome DNA.

The nucleotide sequences of the invention may also be synthetically synthesized by methods known in the art. For example, they may be synthesized by the phosphoramidite procedure on an automated synthesizer  
15 followed by purification by thin layer chromatography as described generally in T.K. Archer et al (1985) J. Biol. Chem. 260:1676-1681. Sequences may also be amplified using the polymerase chain reaction (PCR) which is discussed in more detail below.

20 A number of unique restriction sequences for restriction enzymes are incorporated in the 20 kb sequence, identified in Figure 2, and provide access to novel Y chromosomal nucleotide sequences. It is possible to make use of the recognition sites for restriction enzymes, shown in Figure  
25 2, to prepare partial sequences of the 20 kb sequence. In particular, the 5 kb Eco R1 partial sequence may be subcloned and several nucleotide sequences within the 5 kb partial sequence may be subcloned and sequenced. For example, the nucleotide sequences Y1, Y2 and Y3 are shown  
30 in SEQ ID NO:3, SEQ ID NO:6 and SEQ ID NO:9 and Figures 3, 4 and 5 respectively. DNA fragments unique to Y1, Y2 and Y3 can also be constructed by chemical synthesis and enzymatic ligation reactions carried out in a manner known per se.

35 As hereinbefore mentioned, the invention relates to a

purified and isolated nucleotide sequence having the sequence AUTO 1 shown in SEQ ID NO:12 and Figure 6, or AUTO 2 shown in SEQ ID NO:15 and Figure 7, or a nucleotide sequence having substantial homology thereto which is 5 capable of hybridizing to a significantly greater extent with autosomal DNA than with Y chromosomal DNA.

The invention further provides fragments of the above-mentioned nucleotide sequences, designated AUTO 1 and AUTO 2, preferably comprising the fragments JC4 and JC7 shown 10 in SEQ ID NO:13 and SEQ ID NO:14 respectively and Figure 6 or JK1 and JK2 shown in SEQ ID NO:16 and SEQ ID NO:17 respectively and Figure 7, or sequences having substantial homology thereto.

It is understood that the term "autosomal" refers to 15 genetic material contained in any chromosome other than the Y and X sex chromosomes. The term "sequences having substantial homology thereto" has been previously discussed.

The nucleotide sequences of the invention hybridizing to 20 a significantly greater extent with autosomal DNA may be prepared from bovine genomic DNA, for example by the following method.

Genomic male bovine DNA, may be ligated into a vector, 25 preferably the commercially available vector, PTZ18U and transformed into a prokaryotic or eukaryotic cell, preferably a bacterium, most preferably *E. coli*. The DNA from several transformants may be prepared in quantity and labelled by nick translation. Labelled probes may be selected which hybridize to autosomal DNA present in male 30 and female bovine genomic DNA. Preferably autosomal probes are selected which hybridize to autosomal DNA with approximately the same efficiency as the Y specific probes hybridize to male DNA. The most preferred autosomal nucleotide sequences of the invention AUTO1 and AUTO2 are 35 shown in SEQ ID NO:12 and SEQ ID NO:15 and Figures 6 and

7 respectively.

The nucleotide sequences, sequences having substantial homology thereto and DNA fragments having sequences unique to AUTO1 and AUTO2 can also be constructed by chemical 5 synthesis and enzymatic ligation reactions carried out using methods known in the art as discussed above.

The nucleotide sequences of the invention or fragments of the nucleotide sequences, allow those skilled in the art to construct nucleotide probes for use in the 10 detection of nucleotide sequences in biological materials such as DNA samples, tissues or body fluids. A nucleotide probe may be labelled with a radioactive label which provides for an adequate signal and has sufficient half-life such as  $^{32}\text{P}$ ,  $^3\text{H}$ ,  $^{14}\text{C}$  or the like. Other labels which 15 may be used include antigens that are recognized by a specific labelled antibody, fluorescent compounds, enzymes, antibodies specific for a labelled antigen, and chemiluminescence. An appropriate label may be selected having regard to the rate of hybridization and binding of 20 the probe to the nucleotide to be detected and the amount of nucleotide available for hybridization.

The sequences of the present invention permit the identification and isolation or synthesis of fragments of the nucleotide sequences of the invention which may be 25 used as primers to amplify a corresponding selected nucleotide sequence having a sequence complementary to the primer, for example in the PCR reaction. The present invention also provides pairs of primers which are selected such that they amplify nucleotide sequences of 30 the invention, preferably under hybridizing conditions in the PCR reaction. Primers which may be used to amplify nucleotide sequences which are specific to bovine Y chromosomal DNA include: 5-1 and 6-1 for the sequence Y1, shown in SEQ ID NO:3 and Figure 3; K3 and K4 for the 35 sequence Y2 shown in SEQ ID NO:6 and Figure 4; K1 and K2 for the sequence Y3, shown in SEQ ID NO:9 and Figure 5.

Primers which may be used to amplify sequences which are specific to autosomal DNA include: JC4 and JC7 for the sequence AUTO1, shown in SEQ ID NO:12 and Figure 6; and JK1 and JK2 for the sequence AUTO2, shown in SEQ ID NO:15  
5 and Figure 7.

As hereinbefore mentioned, in a preferred embodiment, the invention relates to a method of determining the sex of a bovine cell, preferably from an embryo, which method comprises isolating a DNA sample from the cell; treating  
10 the sample with at least one pair of hybridization primers which are capable of amplifying the Y-specific sequences of the invention, preferably the pair of hybridization primers 5-1 and 6-1 shown in SEQ ID NO:4 and SEQ ID NO:5 respectively and Figure 3; K3 and K4 shown in SEQ ID NO:7  
15 and SEQ ID NO:8 respectively and in Figure 4; or K1 and K2 shown in SEQ ID NO:10 and SEQ ID NO:11 respectively and Figure 5, under hybridization conditions in the polymerase chain reaction to produce amplified product and; detecting the amplified product, whereby the sex of the cell is  
20 determined as male by the presence of amplified product and female by the absence of amplified product.

In a particularly preferred embodiment the DNA sample is simultaneously treated, as a control, with at least one of the following pairs of hybridization primers: JC4 and JC7  
25 shown in SEQ ID NO:13 and SEQ ID NO:14 respectively and Figure 6 or JK1 and JK2 shown in SEQ ID NO:16 and SEQ ID NO:17 respectively and Figure 7, or primers having substantial homology thereto, which are capable of hybridizing to a significantly greater extent with  
30 autosomal DNA than with Y chromosomal DNA, whereby the presence of bovine DNA and hybridization conditions are confirmed by the presence of autosomal amplification product.

It will be appreciated that the term "hybridization  
35 conditions" refers to reaction conditions which permit hybridization and amplification reactions to proceed in

the presence of the DNA sample and appropriate complementary hybridization primers. Conditions suitable for the polymerase chain reaction are generally known in the art.

5 The term "polymerase chain reaction (PCR)" used herein refers to the process for amplifying a target nucleotide sequence as generally described in Innis et al (ed) PCR Protocols, Academic Press, 1990 and U.S. Patent 4,800,159 to Mullis et al. Amplification of the target nucleotide  
10 sequence may be accomplished by means of a pair of hybridization primers which flank the nucleotide sequence to be amplified. The primers hybridize to opposite strands of the target nucleotide sequence and DNA synthesis proceeds across the region between the  
15 hybridization primers, thereby doubling the amount of that DNA segment. Repeated cycles of denaturation, priming and extension permit rapid exponential amplification of the target nucleotide sequence. The amplified product may then be readily detected by standard techniques, for  
20 example by gel electrophoresis or autoradiography, preferably gel electrophoresis.

The term "hybridization primer(s)" refers to an oligonucleotide isolated and purified from a cell or produced by synthesis, which is capable of hybridizing to  
25 a complementary nucleotide sequence and acting as a point of initiation of synthesis when placed under conditions appropriate for the PCR reaction, in which synthesis of a primer extension product which is complementary to a nucleotide sequence is initiated. The hybridization  
30 primer generally contains 15 to 30 nucleotides and is preferably single stranded. The primers may be synthesized using methods known in the art.

The term "pair(s) of hybridization primers" refers to two hybridization primers which flank the nucleic acid  
35 sequence to be amplified in the PCR reaction. The two hybridization primers are selected so that they

sufficiently hybridize to different strands of the nucleotide sequence to be amplified at relative positions along the sequence such that an extension product synthesized from one primer when it is separated from its 5 complementary strand will serve as a template for the synthesis of an extension product for the other primer. Preferred pairs of hybridization primers of the invention are 5-1 and 6-1, K3 and K4 and K1 and K2. Preferred pairs of hybridization primers which may be used to selectively 10 amplify autosomal DNA are JC4 and JC7, and JK1 and JK2. The DNA sequences of the pairs of hybridization primers are shown in Figures 3, 4, 5, 6 and 7 respectively.

It will be appreciated that a hybridization primer may be modified provided a sufficient amount of the hybridization 15 primer contains a sequence which is complementary to the strand to be amplified. For example, it may be modified to assist in isolating amplified product by introducing a restriction site in the primer.

The DNA sample may be incubated with deoxynucleoside 20 triphosphates (dNTPs), preferably at a concentration of 0.05 to 0.5mM, most preferably 0.2 mM, as well as a pair of bovine Y chromosomal hybridization primers and a pair of control autosomal hybridization primers. To this may be added Taq polymerase, preferably 1 to 5 units, most 25 preferably 2.5 units and the reaction may be carried out preferably for 20 to 70 cycles, most preferably for 45 cycles. In a preferred embodiment, the annealing temperature may be 62°C and the elongation temperature may be 72°C during each cycle. The reactions may be 30 terminated, for example, by the addition of ethanol to precipitate the DNA. The resulting DNA may be analyzed to identify autosomal and Y chromosomal sequences by standard techniques, preferably gel electrophoresis.

The Y chromosomal hybridization primers may be used to 35 accurately determine the sex of animals, preferably bovines, most preferably of the genus Bos, in DNA samples

extracted from a few cells, preferably 3-5 cells. The cells may be obtained from a pre-implantation bovine embryo, preferably a seven day old embryo, or from cells obtained from a sample of amniotic fluid, for example by 5 the following method.

The DNA from several cells of a bovine embryo may be extracted using techniques known in the art and described, for example in Innis et al (ed.), PCR Protocols, Academic Press, 1990, and treated with the Y chromosomal 10 hybridization primers of the invention, preferably with one pair of hybridization primers under hybridization conditions in the PCR reaction, to selectively produce an amplified product i.e. specific sequences on the Y chromosome. Detection of the amplified product indicates 15 a male embryo and the lack of an amplification product indicates a female embryo.

The amplified products may be readily isolated and distinguished by techniques known in the art. Preferably, the amplified products may be distinguished based on their 20 respective sizes using techniques known in the art. For example, after amplification, the DNA sample can be separated on an agarose gel and visualized, after staining with ethidium bromide, under ultra violet (UV) light.

In a preferred embodiment of the invention, the DNA sample 25 from the embryo to be sexed may be simultaneously treated with a pair of Y chromosomal hybridization primers and a pair of autosomal hybridization primers.

In a particularly preferred embodiment the DNA sample may be treated with the following pairs of hybridization 30 primers: JK1 and JK2, which amplify a 113 bp autosomal sequence and K1 and K2 which amplify a 199 bp Y chromosome sequence; or JC4 and JC7 which amplify a 174 bp autosomal sequence and J5-1 and J6-1 which amplify a 700 bp Y chromosome sequence. It is advantageous to use the above- 35 noted pairs of hybridization primers in the particularly

preferred embodiment of the invention as the Y-specific and autosomal pairs of hybridization primers amplify respective products which have significant size differences and which can be readily and clearly separated 5 by size by techniques such as gel electrophoresis. Thus, this particularly preferred embodiment provides for a rapid and clear determination of genetic sex, as illustrated in Figure 8.

The amplification products may be used to determine the 10 sex of the embryo, as illustrated in Figure 8. Amplification of the autosomal sequence acts as a control to confirm the presence of DNA and hybridization primers under hybridization conditions. Amplification of the Y chromosome sequence designates the embryo as male. Lack 15 of amplification of the Y chromosome sequence, combined with amplification of the autosomal sequence designates the embryo as female.

As hereinbefore mentioned, the invention also provides a kit for carrying out the method of sexing bovine cells, 20 comprising a pair of hybridization primers, preferably having the sequences 5-1 and 6-1 shown in SEQ ID NO:4 and SEQ ID NO:5 respectively and Figure 3; K3 and K4 shown in SEQ ID NO:7 and SEQ ID NO:8 respectively and Figure 4; or K1 and K2 shown in SEQ ID NO:10 and SEQ ID NO: 11 25 respectively and Figure 5 or sequences having substantial homology thereto, which under hybridization conditions in the polymerase chain reaction (PCR) amplify a Y chromosomal product, means for detecting the amplified product as discussed above and, the reagents required for 30 the PCR reaction. Preferably, the kit is further composed of a pair of hybridization primers, most preferably the further pair of hybridization primers have the sequences JC4 and JC7 shown in SEQ ID NO:13 and SEQ ID NO:14 respectively and Figure 6 or JK1 and JK2 shown in SEQ ID 35 NO:16 and SEQ ID NO:17 respectively and Figure 7, or sequences having substantial homology thereto, which amplify an autosomal sequence.

It is an advantage of the nucleotide sequences of the present invention that they permit accurate sexing of embryos with a success rate at or close to 100%.

The nucleotide sequences of the invention and fragments thereof are shown in Figures 1 and 3-7 and are also shown in the appended Sequence Listing as follows:

- SEQ ID NO:1 shows the first probe sequence in Figure 1
- SEQ ID NO:2 shows the second probe sequence in Figure 1
- SEQ ID NO:3 shows Y1 in Figure 3
- 10 SEQ ID NO:4 shows 5-1 in Figure 3
- SEQ ID NO:5 shows 6-1 in Figure 3
- SEQ ID NO:6 shows Y2 in Figure 4
- SEQ ID NO:7 shows K3 in Figure 4
- SEQ ID NO:8 shows K4 in Figure 4
- 15 SEQ ID NO:9 shows Y3 in Figure 5
- SEQ ID NO:10 shows K1 in Figure 5
- SEQ ID NO:11 shows K2 in Figure 5
- SEQ ID NO:12 shows AUTO1 in Figure 6
- SEQ ID NO:13 shows JC4 in Figure 6
- 20 SEQ ID NO:14 shows JC7 in Figure 6
- SEQ ID NO:15 shows AUTO2 in Figure 7
- SEQ ID NO:16 shows JK1 in Figure 7
- SEQ ID NO:17 shows JK2 in Figure 7

The following non-limiting examples are illustrative of  
25 the present invention:

Example 1

**Preparation of Bovine Genomic DNA**

DNA prepared according to this Example from male and female bovine tissue is also referred to in the  
30 specification as "male bovine DNA" and "female bovine DNA" respectively.

Male and female bovine liver biopsies were obtained from Holsteins slaughtered at the University of Guelph abattoir. The liver biopsies from each sex were processed

separately by identical procedures to obtain male and female bovine genomic DNA. Bovine genomic DNA was extracted from male and female liver biopsies by the following procedure to produce male bovine DNA and female  
5 bovine DNA.

A 0.25 cm<sup>3</sup> tissue fragment of the liver biopsy was added to 0.7 ml of 50 mM Tris (pH 8.0), 100 mM EDTA, 0.5% SDS. Proteinase K was added to a concentration of 0.5 mg/ml, and the sample incubated at 55°C for 16 hours. Following  
10 incubation, the DNA was extracted with phenol and chloroform generally following the procedure outlined in Sambrook et al, Molecular Cloning, 2nd Ed., Cold Spring Harbor Laboratory Press. Sodium acetate (pH 6.0) was added to a concentration of 0.3M, and the DNA was  
15 collected by precipitation with an equal volume of ethanol at room temperature. The ethanol precipitated DNA was pelleted by centrifugation at 13,000 X g, and the resulting DNA pellets were dissolved in 100 µl of 10 mM Tris, pH 8.0, 1 mM EDTA.

20

Example 2**Construction of Bovine Male Genomic Library and Isolation of Y Chromosome DNA.**

Total male bovine DNA, prepared as described in Example I, was used to construct a bovine male genomic library. A  
25 library of bovine male DNA was constructed in the vector Lambda FIX II (Stratagene), generally following the manufacturer's instructions. The genomic DNA was partially digested with Sau 3A, generating fragments in the range of 10-50 kb. The fragments were ligated to the  
30 lambda arms of the phage vector and the recombinant phage were packaged in vitro following the instructions provided by the manufacturer of the vector.

The resulting library was plated and screened with a DNA probe specific for a 157 bp segment of Y chromosome DNA,  
35 (BOV97M). The Y chromosome DNA probe BOV97M, described by Miller and Koopman (Animal Genetics, 21:77, 1990) was

obtained by PCR amplification from bovine male genomic DNA. The PCR amplification was carried out following the method of Innis et al (PCR Protocols, Academic Press, 1990). The two hybridization primers used in the PCR 5 amplification were prepared using the standard cyanoethyl phosphoramidite synthesis method, and are shown in SEQ ID NOS: 1 and 2 and Figure 1. The hybridization primers are complementary to the ends of the 157 bp segment, BOV97M.

10 After PCR amplification, the DNA band corresponding to BOV97M was purified on a low melting point agarose gel, and ligated into the Sma I site in the polylinker of the cloning vector pTZ18U (Pharmacia). The plasmid was transformed into E. coli strain DH5 $\alpha$ . The identity of the 15 insert was confirmed by DNA sequence analysis, and the BOV97M fragment was prepared in quantity and radiolabelled by nick translation, following the methods of Sambrook et al supra.

The radiolabelled BOV97M fragment was used to screen the 20 bovine male genomic library for positive clones. The labelled fragment was hybridized to a plaque lift of the bovine male genomic library using standard procedures (Sambrook et al, Molecular Cloning, 2nd Edition, Cold Spring Harbor Laboratory Press, 1989). One positive clone 25 was identified. The phage DNA within the positive clone was analyzed and it was found to contain a 20 kilobase pair of Y chromosome DNA, within which was present the 157 bp BOV97M segment. A restriction map of the 20 kb DNA sequence is shown in Figure 2.

30 A 5 kb Eco RI fragment from the 20 kb insert was subcloned in the Eco RI site of pTZ12U, and several fragments within it were subcloned and sequenced. For example, several Bgl II fragments were identified within the 5 kb Eco RI fragment and are shown in Figure 2. The Bgl II fragments 35 Y1, Y2 and Y3, shown in Figure 2, were again subcloned, into the Bam HI site of PTZ18U, and sequenced. The

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nucleotide sequences of the fragments Y1, Y2 and Y3 are shown in SEQ ID NO:3, SEQ ID NO:6 and SEQ ID NO:9 and Figures 3, 4, and 5, respectively.

Example 3

5 Preparation of Hybridization Primers Specific for Male Bovine DNA  
Using the sequence data obtained for the Bgl II fragments, Y1, Y2 and Y3, oligonucleotide hybridization primers for use in PCR amplification of the fragments were chemically  
10 synthesized by the standard dideoxy sequencing method as described by Sambrook et al *supra*. The hybridization primers are complementary to sequences at, or near to, the ends of the fragments. The hybridization primers for the Y1 fragment, designated 5-1 and 6-1 are shown in SEQ ID  
15 NO:4 and SEQ ID NO:5 respectively and are shown underlined on Figure 3; the hybridization primers for the Y2 fragment, designated K3 and K4 are shown in SEQ ID NO:4 and SEQ ID NO:5 respectively and are shown underlined on Figure 4; and the hybridization primers for the Y3  
20 fragment, designated K1 and K2, are shown in SEQ ID NO:10 and SEQ ID NO:11 respectively and are shown underlined on Figure 5.

Example 4

Preparation of Hybridization Primers for Autosomal

25 Bovine DNA  
Male bovine genomic DNA that had been digested with Sau 3A was ligated into the Bam HI site of PTZ18U and transformed into *E. coli*. Plasmid DNA was prepared from several transformants and labelled by nick translation. These  
30 probes were hybridized to male or female genomic DNA that had been spotted onto nitrocellulose. All of the probes tested in this way hybridized to both male and female genomic DNA, indicating that they did not contain Y specific DNA and were from autosomal sequences. This was  
35 not surprising considering that the great majority of genomic DNA in males is also present in females.

The cloned segments in two of these plasmids, AUTO1 and AUTO2, were sequenced and oligonucleotide hybridization primers synthesized that would amplify portions of them in a PCR reaction. The hybridization primers for AUTO1, JC4 5 and JC7 are shown in SEQ ID NO:13 and SEQ ID NO:14 respectively and are shown underlined on Figure 6; and the hybridization primers for AUTO2, JK1 and JK2 are shown in SEQ ID NO:16 and SEQ ID NO:17 respectively and are shown underlined on Figure 7.

10

Example 5Sex Determination of Bovine Embryos

Three to five cells from a bovine embryo to be sexed were removed by micromanipulation from seven day old embryos, and placed into 20  $\mu$ l of sterile H<sub>2</sub>O. The cell samples 15 were either used directly at this stage or were frozen for subsequent analysis. The embryos from which the samples were removed were labelled and maintained in vitro pending the outcome of the sex determination prior to implantation into recipient cows.

20 The sex of the embryo from which the cells were removed was determined by PCR amplification using a combination of bovine male specific hybridization primers and bovine autosomal hybridization primers. Two combinations were most commonly used. One combination was the male specific 25 hybridization primers K1 and K2 with the autosomal hybridization primers JK1 and JK2; and the other was the male specific hybridization primers J5-1 and J6-1 with the autosomal hybridization primers JC4 and JC7.

For analysis, the sample volume was made up to 50  $\mu$ l using 30 PCR buffer (final concentration 50 mM KC1, 10 mM tris, pH 8.4, 1.5 mM MgCl<sub>2</sub>) SDS was added to a final concentration of 2.0  $\mu$ M, and proteinase K to a concentration of 50  $\mu$ g/ml. The sample was then incubated for one hour at 35 55°C, and the proteinase K was then inactivated by heating to 95°C for 10 minutes.

Following incubation, deoxynucleoside triphosphates (dNTPs) were added (0.2 mM final concentration), as well as a set of bovine male specific hybridization primers and a set of control autosomal hybridization primers (0.15 5 µg of male hybridization primers and 0.10 µg of autosomal hybridization primers). The final volume was 100 µl in PCR buffer. To this was added 2.5 units of Taq polymerase, and the reactions were then carried out for 45 cycles using an annealing temperature of 62°C and an 10 elongation temperature of 72°C during each cycle. The reactions were terminated by the addition of two volumes of ethanol to precipitate the DNA.

The DNA was pelleted by centrifugation. The DNA pellets were resuspended in 15 µl of gel loading buffer (3% 15 Ficoll, 0.03% xylene cyanol), and run on a 1.5% agarose gel. The gel was then stained in ethidium bromide and the DNA visualized on a UV light transilluminator. The sex of the embryo was determined as shown in Figure 8. The embryo was determined to be female based upon the 20 presence of one amplified band on the gel, representing the autosomal sequence. The embryo was determined to be male based upon the presence of two amplified bands on the gel, representing the male specific sequence and the autosomal sequence. Polaroid photographs of the gel were 25 taken to provide a permanent record of the results.

To confirm the sex of the embryo tested by the above PCR amplification reaction the embryos were transferred to recipient cows and carried to term.

A total of over 100 embryos have been sexed by means of 30 the above PCR reaction and 89 sexed embryos have been transferred to recipient cows. Sixteen live calves have been produced and the sex of the embryos as determined by the PCR reaction of the present invention was confirmed in all cases by the sex of calf produced.

35 The present invention has been described in detail and

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with particular reference to the preferred embodiments; however, it will be understood by one having ordinary skill in the art that changes can be made thereto without departing from the spirit and scope thereof.

- 5 Forming part of the present disclosure are the appended sequence listings.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Wildeman, Alan G.  
Kelly, John J.
- (ii) TITLE OF INVENTION: Nucleotide sequences for bovine sex determination
- (iii) NUMBER OF SEQUENCES: 17
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Linda M. Kurdydyk, Bereskin & Parr
  - (B) STREET: Box 401, 40 King Street West
  - (C) CITY: Toronto
  - (D) STATE: Ontario
  - (E) COUNTRY: Canada
  - (F) ZIP: M5H 3Y2
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Kurdydyk, Linda M.
  - (B) REGISTRATION NUMBER: 34,971
  - (C) REFERENCE/DOCKET NUMBER: 6580-003
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: (416) 361-7311
  - (B) TELEFAX: (416) 361-1398
  - (C) TELEX: 06-23115

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 26 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: Bos taurus
- (viii) POSITION IN GENOME:
  - (A) CHROMOSOME SEGMENT: Y Chromosome

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GATCACTATA CATAACCAC TCTCAT

26

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 26 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:  
(A) ORGANISM: Bos taurus

(viii) POSITION IN GENOME:  
(A) CHROMOSOME SEGMENT: Y Chromosome

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TGATAAAAAG GCTATGCTAC ACAAAAT

26

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 745 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: double  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:  
(A) ORGANISM: Bos taurus  
(B) STRAIN: Holstein  
(P) TISSUE TYPE: Liver

(vii) IMMEDIATE SOURCE:  
(A) LIBRARY: Bovine male DNA library in lambda FIX II  
(Stratagene)  
(B) CLONE: Y1

(viii) POSITION IN GENOME:  
(A) CHROMOSOME SEGMENT: Y Chromosome

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GATCTTGGGA GCAAGCAAAG TGTGCGCCA CCTGAATAAT GTTGCCTAAA ATTTTATTT 60

GACATATCCA CATAACATACC CATGTGTGTA TGAGTTGGG TATAAAAAAA TTACATTGAC 120

TTTTAATTCC CATTAGGTAT AACAAACATGG AAAAATTTAA ACCCAAATGC AGAATGACTC 180

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AAGACTCTAC ACAGTTATTG AATATATGTA AGTGGAAATA CTTCAAAATA TGTTTATTG	240
GCTACATCTA ACATATAAGG ACTCTTTAT ACCACTCCC CTCTCTCCTT TTAATAAATA	300
CCTACCAATT TTCAAAGTTT GCCCAGGTTT ATGAAATACT CAAGGAATAA TAGGAAAAT	360
ATATATATAT ATATATTATA TATATATATA GGAGAGGTAG GAACCAATCA TTACTCTGCT	420
GGCCAATCCA TGGATGGAGG AGCTGGTAG CCTGCAGTC ATGGTGTAC AAAATAGATC	480
GGTACAAC TGAGTCACTCC CTTCACTTT TCACCTTCAT GCATTGGAGA AGGACATAGC	540
AACCCACTCG AATGTTCTTG CCTGGAGAAT CCTGGGACAG ATGAGCCTGG TGGGCTGCCG	600
TCTGTGGGT CACACAGAGT TGGACACGTC TGAAATGACA GCAGATATAC CTATTTATAT	660
GCATATTTAT GTATATAGAT ATACAAATAT CTATATAAAA TAGATATGTT TCCATTGCAG	720
AAAATGTTGA TACCAACCTT AGATC	745

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Bos Taurus
- (B) STRAIN: Holstein
- (F) TISSUE TYPE: Liver

(vii) IMMEDIATE SOURCE:

- (A) LIBRARY: Bovine male DNA library in lambda FIX II
- (B) CLONE: Y1

(viii) POSITION IN GENOME:

- (A) CHROMOSOME SEGMENT: Y Chromosome

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CACCTGAATA ATGTTGCCTA AAATT	25
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(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Bos taurus  
 (B) STRAIN: Holstein  
 (F) TISSUE TYPE: Liver

(vii) IMMEDIATE SOURCE:  
 (A) LIBRARY: Bovine male DNA library in lambda FIX II  
 (B) CLONE: Y1

(viii) POSITION IN GENOME:  
 (A) CHROMOSOME SEGMENT: Y Chromosome

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TTTCTGCAAT GGAAACATAT CTATT

25

## (2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 257 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: double  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:  
 (A) ORGANISM: Bos taurus  
 (B) STRAIN: Holstein  
 (F) TISSUE TYPE: Liver

(vii) IMMEDIATE SOURCE:  
 (A) LIBRARY: Bovine male DNA library in lambda FIX II  
 (B) CLONE: Y2

(viii) POSITION IN GENOME:  
 (A) CHROMOSOME SEGMENT: Y Chromosome

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GATCTCAGAA TTATTCCAA CACCATGCTC CAGGTCAACA GGAAATAGCC AAAGTTGTTG	60
CTGACACAAA CAGTCAAGA GCTGGGAAGC CTGTAGACCT CCTCATTTC CAAGAGTAAT	120
TTAACAGAG AAGCTAGAAA TCCAGAAATT AAGGAAAATA ATCAAGAAC AGAAGATAGT	180
AATGATACTT TCACTTTAA CTTTCATGCA TTGGAGAAGG AAATGGCAAC CCATTCCAGT	240
GTTCTCCCT GGAGAAT	257

## (2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 26 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Bos taurus
- (B) STRAIN: Holstein
- (F) TISSUE TYPE: Liver

(vii) IMMEDIATE SOURCE:

- (A) LIBRARY: Bovine male DNA library in lambda FIX II
- (B) CLONE: Y2

(viii) POSITION IN GENOME:

- (A) CHROMOSOME SEGMENT: Y Chromosome

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GATCTCAGAA TTATTCCAA CACCAT

26

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Bos taurus
- (B) STRAIN: Holstein
- (F) TISSUE TYPE: Liver

(vii) IMMEDIATE SOURCE:

- (A) LIBRARY: Bovine male DNA library in lambda FIX II
- (B) CLONE: Y2

(viii) POSITION IN GENOME:

- (A) CHROMOSOME SEGMENT: Y Chromosome

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ATTCTCCAGG GAAGAACACT GGAAT

25

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 199 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Bos taurus

(B) STRAIN: Holstein  
 (F) TISSUE TYPE: Liver

## (vii) IMMEDIATE SOURCE:

(A) LIBRARY: Bovine male DNA library in lambda FIX II  
 (B) CLONE: Y3

## (viii) POSITION IN GENOME:

(A) CHROMOSOME SEGMENT: Y Chromosome

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ATAGATCTCA AAACTGAGTC CCTAGTAAGT CTATAATCAT ACAAAAGAAGA TCTAACGGAG	60
GTTTCTGAAC CAGTTGATTG ATCCTTAGGA GTCCCATCTT GAATTCTGA AGAAATATGT	120
GCCATCTCAA AGAAAGTCTT TCTTCAGATA TTTTCTTATT TGTCTGATTT ACACACTGCA	180
ACTTCAAATG CTGCTTGGC	199

## (2) INFORMATION FOR SEQ ID NO:10:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 26 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (vi) ORIGINAL SOURCE:

(A) ORGANISM: Bos taurus  
 (B) STRAIN: Holstein  
 (F) TISSUE TYPE: Liver

## (vii) IMMEDIATE SOURCE:

(A) LIBRARY: Bovine male DNA library in lambda FIX II  
 (B) CLONE: Y3

## (viii) POSITION IN GENOME:

(A) CHROMOSOME SEGMENT: Y Chromosome

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ATAGATCTCA AAACTGAGTC CCTAGT	26
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## (2) INFORMATION FOR SEQ ID NO:11:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Bos taurus
- (B) STRAIN: Holstein
- (F) TISSUE TYPE: Liver

(vii) IMMEDIATE SOURCE:

- (A) LIBRARY: Bovine male DNA library in lambda FIX II
- (B) CLONE: Y3

(viii) POSITION IN GENOME:

- (A) CHROMOSOME SEGMENT: Y Chromosome

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GCCAAGCAGC ATTTGAAGTT GCAGT

25

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 174 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Bos taurus
- (B) STRAIN: Holstein
- (F) TISSUE TYPE: Liver

(vii) IMMEDIATE SOURCE:

- (A) LIBRARY: Bovine male DNA library in lambda FIX II
- (B) CLONE: AUTO1

(viii) POSITION IN GENOME:

- (A) CHROMOSOME SEGMENT: Autosome

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ACCAAGACTTC TATAGTTGAA ACCTGTGCTG GATTCTGAGA GCAAGAAACC TCCACCGCC 60

TGATAAAAACT GCATGGGAGA GTTGGTTCCA GACTGAACAA TGTGAGCATG GAAAAGCCAT 120

TCTTCTAATT AGATTGGCAG ACAGGAACAA CCTCTGCTAT GAGGTGAAAT ACAC 174

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

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(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Bos taurus
- (B) STRAIN: Holstein
- (F) TISSUE TYPE: Liver

(vii) IMMEDIATE SOURCE:

- (A) LIBRARY: Bovine male DNA library in lambda FIX II
- (B) CLONE: AUTO1

(viii) POSITION IN GENOME:

- (A) CHROMOSOME SEGMENT: Autosome

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

ACCAAGACTTC TATAGTTGAA ACCTGT

26

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Bos taurus
- (B) STRAIN: Holstein
- (F) TISSUE TYPE: Liver

(vii) IMMEDIATE SOURCE:

- (A) LIBRARY: Bovine male DNA library in lambda FIX II
- (B) CLONE: AUTO1

(viii) POSITION IN GENOME:

- (A) CHROMOSOME SEGMENT: Autosome

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GTGTATTCA CCTCATAGCA GAGGTT

26

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 129 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Bos taurus
- (B) STRAIN: Holstein

## (vii) IMMEDIATE SOURCE:

- (A) LIBRARY: Bovine male DNA library lambda FIX II
- (B) CLONE: AUTO2

## (viii) POSITION IN GENOME:

- (A) CHROMOSOME SEGMENT: Autosome

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GATCGAACTG ATGGTCCCTA TATTGCCAGG TGGATTCTTC ACTACTGGAC CACCAGGGAA	60
GTCCCTGATG TGTTTTTTTG TTTTTTTTT TTTTAAACTG AAGGATAATG CAGCTAAAAT	120
CAGCAGATC	129

## (2) INFORMATION FOR SEQ ID NO:16:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: Bos taurus
- (B) STRAIN: Holstein
- (F) TISSUE TYPE: Liver

## (vii) IMMEDIATE SOURCE:

- (A) LIBRARY: Bovine male DNA library in lambda FIX II
- (B) CLONE: AUTO2

## (viii) POSITION IN GENOME:

- (A) CHROMOSOME SEGMENT: Autosome

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GAACTGATGG TCCCTATATT GCCAG	25
-----------------------------	----

## (2) INFORMATION FOR SEQ ID NO:17:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (vi) ORIGINAL SOURCE:

- (A) ORGANISM: Bos taurus
- (B) STRAIN: Holstein
- (F) TISSUE TYPE: Liver

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(vii) IMMEDIATE SOURCE:

- (A) LIBRARY: Bovine male DNA library in lambda FIX II
- (B) CLONE: AUTO2

(viii) POSITION IN GENOME:

- (A) CHROMOSOME SEGMENT: Autosome

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TTAGCTGGAT TATCCTTCAC TTTAA

25

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A purified and isolated nucleotide sequence comprising the sequence Y1 shown in SEQ ID NO:3; Y2 shown in SEQ ID NO:6; or Y3 shown in SEQ ID NO:9, which is capable of hybridizing to a significantly greater extent with Y chromosomal DNA than with autosomal or X chromosomal DNA.
2. A purified and isolated nucleotide sequence as claimed in claim 1 comprising the sequence Y1 shown in SEQ ID NO:3.
3. A purified and isolated nucleotide sequence as claimed in claim 1 comprising the sequence Y2 shown in SEQ ID NO:6.
4. A purified and isolated nucleotide sequence as claimed in claim 1 comprising the sequence Y3 shown in SEQ ID NO:9.
5. A nucleotide sequence having substantial homology to the nucleotide sequence as claimed in claim 1.
6. A fragment of the nucleotide sequence as claimed in claim 1 or any contiguous portion thereof consisting of 15 or more nucleotides.
7. A fragment as claimed in claim 6 comprising the sequence 5-1 or 6-1 shown in SEQ ID NO:4 and SEQ ID NO:5; K3 or K4 shown in SEQ ID NO:7 and SEQ ID NO:8; or K1 or K2 shown in SEQ ID NO:10 and SEQ ID NO:11 or a sequence having substantial homology thereto.
9. A purified and isolated nucleotide sequence having the sequence AUTO 1 shown in SEQ ID NO:12, or AUTO 2 shown in SEQ ID NO:15, which is capable of hybridizing to a significantly greater extent with autosomal chromosomal DNA than with Y chromosomal DNA.

10. A fragment of the nucleotide sequence claimed in claim 9 or any contiguous portion thereof consisting of 15 or more nucleotides.

11. A fragment as claimed in claim 10 comprising the 5 sequence JC4 or JC7 shown in SEQ ID NO:13 and SEQ ID NO:14 or JK1 or JK2 shown in SEQ ID NO:16 and SEQ ID NO:17, or a sequence having substantial homology thereto.

12. A method of determining the sex of a bovine cell, which method comprises isolating a DNA sample from the 10 cell; treating the sample with one or more pairs of hybridization primers, which are capable of amplifying a sequence as claimed in claim 1 or 5, under hybridization conditions in the polymerase chain reaction to produce amplified product and; detecting the amplified product, 15 whereby the sex of the cell is determined as male by the presence of amplified product and female by the absence of amplified product.

13. A method as claimed in claim 12 wherein the pairs of hybridization primers comprise 5-1 and 6-1 shown in SEQ ID 20 NO:4 and SEQ ID NO:5; K3 and K4 shown in SEQ ID NO:7 and SEQ ID NO:8; or K1 and K2 shown in SEQ ID NO:10 and SEQ ID NO:11.

14. A method as claimed in claim 13 whereby as a control the DNA sample is simultaneously treated with one or more 25 pairs of hybridization primers comprising JC4 and JC7 shown in SEQ ID NO:13 and SEQ ID NO:14 or JK1 and JK2 shown in SEQ ID NO:16 and SEQ ID NO:17, or sequences having substantial homology thereto, which are capable of hybridizing to a significantly greater extent with 30 autosomal DNA than with Y chromosomal DNA, whereby the presence of bovine DNA and hybridization conditions are confirmed by the presence of autosomal amplification product.

15. A method as claimed in claim 12 wherein the bovine

cell is from an embryo.

16. A kit for determining the sex of a bovine cell comprising a pair of hybridization primers which are complementary to a fragment of the nucleotide sequence 5 Y1 shown in SEQ ID NO:3, Y2 shown in SEQ ID NO:6; or Y3 shown in SEQ ID NO:9, and which are capable of hybridizing to a significantly greater extent with Y chromosomal DNA than with autosomal or X chromosomal DNA under hybridization conditions in the polymerase chain reaction 10 (PCR) to produce a Y chromosomal amplification product; the reagents required for the PCR reaction and; means for detecting the amplification product.

17. A kit as claimed in claim 16 which additionally 15 comprises a pair of hybridization primers which are complementary to a fragment of the nucleotide sequence, AUTO 1 shown in SEQ ID NO:12, or AUTO 2 shown in SEQ ID NO:15, and which are capable of hybridizing to a significantly greater extent with autosomal chromosomal 20 DNA than with Y chromosomal DNA under hybridization conditions in the polymerase chain reaction (PCR) to produce an autosomal amplification product.

18. A kit as claimed in claim 16 for use in determining the sex of bovine embryo cells.

25 19. A method of detecting the presence of Y chromosomal DNA in a tissue or cell sample comprising selecting a fragment as claimed in claim 6 labelled with a detectable marker, contacting the sample under hybridization conditions with the fragment and determining the degree of 30 hybridization between the DNA of the sample and the fragment.

FIGURE 1

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5'-GATCACTATAACATAACCACTCTCAT-3'

5'-TGATAAAAAGGCTATGCTACACAAAT-3'

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FIGURE 2

BOV97M is a 157 bp Sau 3A fragment contained within the 350 bp Bgl II fragment  
It is this fragment that was cloned by Miller and Koopman, and published by them

E - Eco RI  
B - Bgl II  
S - Sau 3A

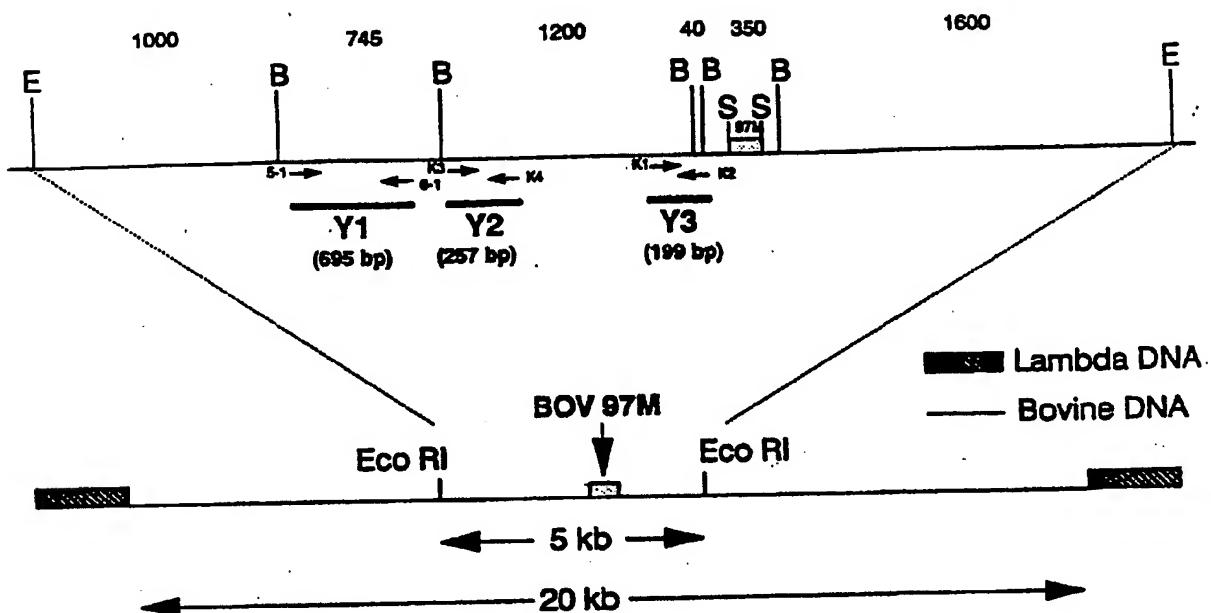


FIGURE 3

2113957

## \*\*\* INPUT INFORMATION \*\*\*

FILE: Y1.SEQ

SEQUENCE: 745BP; 249 A; 134 C; 133 G;

## \*\*\* SEQUENCE LIST \*\*\*

(DOUBLE) 5-1 (25-mer)

	10	20	30	40	50	60
5'	GATCTTGGGA	GCAAGCAAAG	TGTTTGGCCA	CCTGAAATAAT	GTTCGCCTAAA	ATTTTATT
3'	CTAGAACCT	CGTTGTTTC	ACAAACCGGT	GGACTTATTA	CAACGGATT	AAAATAAAA
	70	80	90	100	110	120
	GACATATCCA	CATACATACC	CATGTGTGTA	TGAGTTGGG	TATAA	TTACATTGAC
	CTGTATAGGT	GTATGTATGG	GTACACACAT	ACTCAAAACCC	ATATTTTTT	AATGTAAC
	130	140	150	160	170	180
	TTTTAATTCC	CATTAGGTAT	AAACAAACATGG	AAAAATTAAA	ACCCAAATGC	AGAATGACTC
	AAAATTAAAGG	GTAATCCATA	TTGTTGTACC	TTTTTAATT	TGGGTTTACG	TCTTACTGAG
	190	200	210	220	230	240
	AAGACTCTAC	ACAGTTATTG	AAATATATGTA	AGTGGAAATA	CTTCMAATA	TGTTTTATTG
	TTCTGAGATG	TGTCATAAAC	TTATATACAT	TCACCTTAT	GAAGTTTAT	ACAAATAAC
	250	260	270	280	290	300
	GCTACATCTA	ACATATAAGG	ACTCTTTT	ACCACTTCCC	CTCTCTCCTT	TTAATAATA
	CGATGTAGAT	TGTATATTCC	TGAGAAAATA	TGGTGAAGGG	GAGAGAGGAA	AATTATTTAT
	310	320	330	340	350	360
	CCTACCAATT	TTCAAAGTTT	GCCCAGGTTT	ATGAAATACT	CAAGGAATAA	TAGGAAAAAT
	GGATGGTTAA	AAGTTTCAAA	CGGGTCCAAA	TACTTATGA	GTTCCATTATT	ATCCTTTTA
	370	380	390	400	410	420
	ATATATATAT	ATATATTATA	TATATATATA	GGAGAGGTAG	GAACCAATCA	TTACTCTGCT
	TATATATATA	TATATAATAT	ATATATATAT	CCTCTCCATC	CTTGGTTAGT	AATGAGACGA
	430	440	450	460	470	480
	GGCCAATCCA	TGGATGGAGG	AGCCTGGTAG	GCTGCAGTCC	ATGGTGTAC	AAAATAGATC
	CCGGTTAGGT	ACCTACCTCC	TCGGACCATC	CGACGTCAGG	TACCACAGTG	TTTATCTAG
	490	500	510	520	530	540
	GGTACAACTG	AGTGAATTCC	CTTTCACTTT	TCACTTTCAT	GCATTGGAGA	AGGACATAGC
	CCATGTTGAC	TCACTGAAGG	AAAAGTGA	AGTGAAGA	CGTAACCTCT	TCCTGTATCG
	550	560	570	580	590	600
	AACCCACTCG	AATGTTCTTG	CCTGGAGAAT	CCTGGGACAG	ATGAGCCTGG	TGGGCTGCCG
	TTGGGTGAGC	TTACAAGAAC	GGACCTCTTA	GGACCCCTGTC	TACTCGGACC	ACCCGACGGC
	610	620	630	640	650	660
	TCTGTGGGGT	CACACAGAGT	TGGACACGTC	TGAAATGACA	GCAGATATAC	CTATTTATAT
	AGACACCCCA	GTGTGTCTCA	ACCTGNGCAG	ACTTTACTGT	CGTCTATATG	GATAAAATATA
	670	680	690	700	710	720
	GCATATTAT	GTATATAGAT	ATACAAATAT	CTATATAAAA	TAGATATGTT	TCCATTGAG
	CGTATAATA	CATATATCTA	TATGTTATA	GATATAT	ATCTAAACAA	AGGTAACGTC
	730	740				
	AAAATGTTGA	TACCAACCTT	AGATC 3'			
	TTTATCAACT	ATGGTTGGAA	TCTAG 5'			

↑  
6-1 (25-mer)

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## \*\*\* INPUT INFORMATION \*\*\*

FILE: Y2.SEQ

SEQUENCE: 257BP; 92 A; 52 C; 48 G;

\*\*\* SEQUENCE LIST \*\*\*  
K3 (26-mer)

(DOUBLE)

10	↓	20	30	40	50	60
5'	GATCTCAGAA	TTATTTCCAA	CACCATGCTC	CAGGTCAACA	GGAAATTAGCC	AAAGTTGTTG
3'	CTAGAGTCTT	AATAAAGGTT	GTGGTACGAG	GTCCAGTTGT	CCTTTATCGG	TTTCAACAAAC
	70	80	90	100	110	120
	CTGACACAAA	CAGTTCAAGA	GCTGGGAAGC	CTGTAGACCT	CCTCATTTC	CAAGAGTAAT
	GACTGTGTTT	GTCAAGTTCT	CGACCCCTCG	GACATCTGGA	GGAGTAAAG	GTTCTCATTA
	130	140	150	160	170	180
	TTAACATCAGAG	AAGCTAGAAA	TCCAGAAAATT	AAGGAAAATA	ATCAAGCAAC	AGAAGATAGT
	AATTAGTCTC	TTCGATCTTT	AGGTCTTTAA	TTCCTTTAT	TAGTTCGTTG	TCTCTATCA
	190	200	210	220	230	240
	AATGATAACTT	TCACCTTTAA	CTTTCATGCA	TTGGAGAAGG	AAATGGCAAC	CCATTCCAGT
	TTACTATGAA	AGTGAAAATT	GAAAGTACGT	AACCTCTTCC	TTTACCGTTG	GGTAAGGTCA
	250					
	GTTCTTCCCT	GGAGAAAT	3'			
	CAAGAAGGGA	CCTCTTA	5'			

↑  
K4 (25-mer)

FIGURE 5

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## \*\*\* INPUT INFORMATION \*\*\*

FILE: Y3.SEQ

SEQUENCE: 199BP; 61 A; 39 C; 33 G;

## \*\*\* SEQUENCE LIST \*\*\*

(DOUBLE)

K1 (26-mer)

10	↓	20	30	40	50	60
5'	ATAGATCTCA	AAXCTGAGTC	CCTAGTAAAGT	GTATAATCAT	ACAAAGAAGA	TCTAACGGAG
3'	TATCTAGAGT	TTTGA	CTCAG	GGATCATTC	TGTTTCTTCT	AGATTGCCTC
	70	80	90	100	110	120
	GTTTCTGAAC	CAGTTGATT	ATCCCTAGGA	GTCCCACCTT	GAATTTCTGA	AGAAATATGT
	CAAAGACTTG	GTCAACTAAG	TAGGAATCCT	CAGGGTAGAA	CTTAAAGACT	TCTTTATACA
	130	140	150	160	170	180
	GCCATCTCAA	AGAAAGTCTT	TCTTCAGATA	TTTTCTTATT	TGTCTGATTT	ACACACTGCA
	CGGTAGAGTT	TCTTCAGAA	AGAAGTCTAT	AAAAGAATAA	ACAGACTAAA	TGTGTGACGT
	190					
	ACTTCAAATG	CTGCTTGGC	3'			
	<u>TGAAGTTAC</u>	<u>GACGAACCG</u>	5'			

↑  
K2 (26-mer)

FIGURE 6

2113957

## \*\*\* INPUT INFORMATION \*\*\*

FILE: AUTO1.SEQ

SEQUENCE: 174BP; 57 A; 37 C; 40 G;

## \*\*\* SEQUENCE LIST \*\*\*

(DOUBLE)

JC4 (26-mer)

10	↓	20	30	40	50	60
5'	ACCAGACTTC	TATACTTGA	ACCTGGTGCTG	GATTCTGAGA	GCAAGAAACC	TCCACCCAGCC
3'	TGGTCTGAAG	ATATCAACTT	TGGACACGAC	CTAAGACTCT	CGTTCTTGG	AGGTGGTCGG
	70	80	90	100	110	120
	TGATAAAA	ACTGGGAGA	GTTGGTCCA	GACTGAACAA	TGTGAGCATG	GAAAAGCCAT
	ACTATTTGA	CGTACCCCTCT	CAACCAAGGT	CTGACTTGT	ACACTCGTAC	CTTTTCGGTA
	130	140	150	160	170	
	TCTTCTAATT	AGATTGGCAG	ACAGGAACAA	CCTCTGCTAT	GAGGTGAAT	ACAC 3'
	AGAAGATTAA	TCTAACCGTC	TGTCTTGT	GGAGACGATA	CTCCACFTTA	TGTG 5'

↑  
JC7 (26-mer)

FIGURE 7 2113957

\*\*\* INPUT INFORMATION \*\*\*

FILE: AUTO2.SEQ SEQUENCE: 129BP; 32 A; 23 C; 28 G;

\*\*\* SEQUENCE LIST \*\*\* JK1 (25-mer) (DOUBLE)

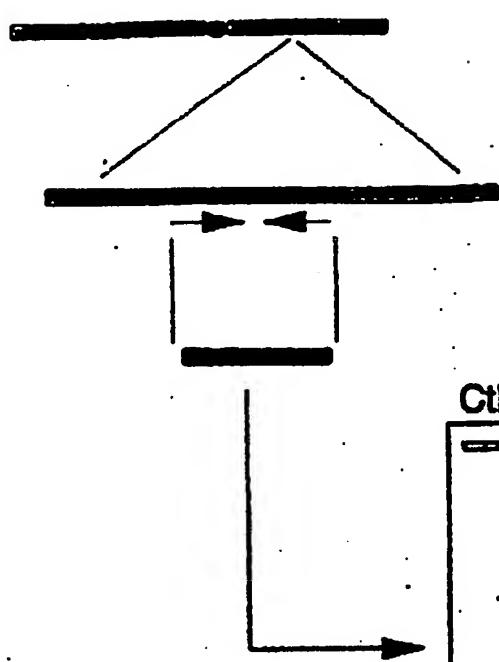
10 ↓ 20 30 40 50 60  
5' GATCGAACTG ATGGTCCCTA TATTGCCAGG TGGATTCTTC ACTACTGGAC CACCAGGGAA  
3' CTAGCTTGAC TACCAAGGGAT ATAACGGTCC ACCTAAGAAG TGATGACCTG GTGGTCCCTT  
70 80 90 100 110 120  
GTCCCCTGATG TGTGTGTGTG TTTTTTTTTT TTTTAAGTG AAGGATAATG CAGCTAAAAT  
CAGGGACTAC ACAAAAAAAC AAAAAAAAATG TTCTTAC GTCGATTITA  
CAGCAGATC 3'  
GTCGTCTAG 5'

JK2 (25-mer)

FIGURE 8

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**Autosome**



**Y chromosome**

